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Invited Minireview—

Recent Advances in Immunomodulation and Vaccination Strategies Against Coccidiosis

Rami A. Dalloul and Hyun S. Lillehoj[A]

Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, USDA-ARS, BARC-East, Building 1040, Beltsville, MD 20705

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SUMMARY. Coccidiosis is a ubiquitous intestinal protozoan infection of poultry seriously impairing the growth and feed utilization of infected animals. Conventional disease control strategies rely heavily on chemoprophylaxis, which is a tremendous cost to the industry. Existing vaccines consist of live virulent or attenuated *Eimeria* strains with limited scope of protection against an ever-evolving and widespread pathogen. The continual emergence of drug-resistant strains of *Eimeria*, coupled with the increasing regulations and bans on the use of anticoccidial drugs in commercial poultry production, urges the need for novel approaches and alternative control strategies. Because of the complexity of the host immunity and the parasite life cycle, a comprehensive understanding of host-parasite interactions and protective immune mechanisms becomes necessary for successful prevention and control practices. Recent progress in functional genomics technology would facilitate the identification and characterization of host genes involved in immune responses as well as parasite genes and proteins that elicit protective host responses. This study reviews recent coccidiosis research and provides information on host immunity, immunomodulation, and the latest advances in live and recombinant vaccine development against coccidiosis. Such information will help magnify our understanding of host-parasite biology and mucosal immunology, and we hope it will lead to comprehensive designs of nutritional interventions and vaccination strategies for coccidiosis.

RESUMEN. Estudio recapitulativo por invitación. Avances recientes en estrategias de modulación inmunológica y vacunación contra la coccidiosis.

La coccidiosis es una infección protozoaria intestinal ampliamente distribuida en las aves que afecta seriamente el crecimiento y la absorción de nutrientes alimenticios en los animales infectados. Las estrategias convencionales para el control de la enfermedad dependen de la quimioprofilaxis, la cual constituye un enorme costo para la industria avícola. Las vacunas existentes constan de cepas vivas de *Eimeria*, virulentas o atenuadas, con un espectro de protección limitado contra cepas de *Eimeria* caracterizadas por su constante evolución y amplia distribución. La aparición continua de cepas de *Eimeria* resistentes a las drogas, sumada al aumento de las regulaciones y prohibiciones en el uso de drogas anticoccidiales en la producción avícola comercial, aumentan la necesidad de establecer nuevas alternativas y estrategias de control. Debido a lo complejo de la inmunidad del huésped y del ciclo de vida del parásito, es necesario tener un amplio conocimiento de las interacciones huésped-parásito y de los mecanismos de protección inmune para el establecimiento de prácticas exitosas de prevención y control. Los progresos recientes en tecnología genética funcional facilitarán la identificación y caracterización de los genes del huésped involucrados en las respuestas inmunes, al igual que los genes y las proteínas del parásito que desencadenan las respuestas inmunes protectoras en el huésped. En este estudio se revisa la investigación reciente sobre coccidiosis y se suministra información sobre la inmunidad del huésped, modulación inmunológica y los últimos avances en el desarrollo de vacunas vivas y vacunas recombinantes contra la coccidiosis. Dicha información aumentará nuestro conocimiento sobre la biología huésped-parásito, la inmunología a nivel de la mucosa intestinal, esperando que conducirá al desarrollo comprensivo de estrategias nutricionales y de vacunación contra la coccidiosis.

Key words: coccidiosis, recombinant vaccines, live vaccines, immunomodulation, *Eimeria*, intestinal immunity, *in ovo*, anticoccidials

Abbreviations: CMI = cell-mediated immunity; DNA = deoxyribonucleic acid; EST = expressed sequence tag; IEL = intraepithelial lymphocyte; IFN = interferon; Ig = immunoglobin; IL = interleukin; ODN = oligodeoxynucleotide; QTL = quantitative trait loci; TGF = transforming growth factor

Avian coccidiosis is the major parasitic disease of poultry, with substantial economic burden estimated to cost the industry more than $800 million in annual losses (89). In-feed medication for prevention and treatment contributes a major portion of those costs. Losses are also attributed to mortality, malabsorption, inefficient feed utilization, impaired growth rate in broilers, and a temporary reduction of egg production in layers. Coccidiosis is caused by several apicomplexan parasites of the genus *Eimeria* that infect the intestinal tract and are transmitted among birds via ingestion of infective oocysts during feeding. *Eimeria* spp. possess a complex life cycle consisting of both sexual and asexual stages, they are host and infection-site specific, and their pathogenicity varies in birds of different genetic background (31,43,55). Therefore, in the natural host, the immunity is species specific (e.g., chickens immune to one species of *Eimeria* are susceptible to others). Additionally, *Eimeria* spp. exhibit different tissue and organ specificity in the infected host. Understanding the interplay between the host and the parasites in the intestine is crucial for the design of novel control approaches against coccidiosis.

Although natural infection with *Eimeria* spp. induces immunity, vaccination procedures on a commercial scale have shown limited effectiveness, and disease control remains largely dependent on routine use of anticoccidial drugs (2,90). Available live vaccines are composed of either virulent or attenuated strains, but a major

[A]Corresponding author.

disadvantage is that the large number of live parasites makes them relatively laborious and costly to produce. Although live oocyst vaccines represent a limited but useful alternative to anticoccidial drugs, a vaccine composed of parasite antigens and antigen-encoding genes that elicit specific immunity is eminently preferable. And although it might be cost effective to produce recombinant vaccines (proteins or deoxyribonucleic acid [DNA]), the difficulty remains to identify which antigens or genes are responsible for eliciting protective immunity or how these recombinant vaccines should be delivered and presented to the bird's immune system. Also, such subunit vaccines could alleviate the danger of emerging resistant strains encountered with live vaccines, but until efficient vaccines become commercially available the poultry industry is forced to rely upon prophylactic chemotherapy to control the disease. Furthermore, the introduction of alternative prevention and treatment measures such as nonchemical feed supplements that effectively enhance productivity and nonspecific immunity may help limit the use of anticoccidials. However, the lack of efficient vaccines, the increasing incidence of drug-resistant strains, and the escalating public anxiety over chemical residues in meat and eggs mandate the development of alternative control methods.

HOST IMMUNITY TO *EIMERIA*

Given that *Eimeria* parasites exhibit a complex life cycle composed of intracellular, extracellular, asexual, and sexual stages, it is not surprising that host immune responses are also complex. Immune responses to this pathogen involve many facets of nonspecific and specific immunity (69,71), the latter encompassing both cellular and humoral immune mechanisms (45,52,53). Nonspecific factors include physical barriers, phagocytes and leukocytes, and complement; specific immunity is mediated by antibodies, lymphocytes, and cytokines. In general, the gut-associated lymphoid tissues serve three functions in host defense against enteric pathogens: processing and presentation of antigens, production of intestinal antibodies, and activation of cell-mediated immunity (CMI). In immune hosts, parasites enter the gut early after infection but are prevented from further development, indicating that acquired immunity to coccidiosis may involve mechanisms that inhibit the natural progression of parasite development (70,80). Recent studies demonstrated the role of several cytokines produced locally during coccidiosis (65), which were responsible for enhancing protective immunity against *Eimeria* (46,52,53,56,94,95).

A concrete role of humoral immunity in the fight against poultry coccidiosis is yet to be defined. Similar to mammals, three classes of antibodies are recognized in birds—immunoglobin (Ig)M, IgA, and IgY—which is considered the orthologue of the mammalian IgG (41) even though the complementary DNA (cDNA) encoding the IgY heavy chain is similar to mammalian IgE (68). The presence of other antibody classes such as IgD or IgE in birds has not been documented. The role of parasite-specific antibodies in both serum and mucosal secretions has been extensively studied in coccidiosis (12,25,54,56,66,78). Upon exposure to *Eimeria* spp., chickens produce all three classes of antibodies. Maternal IgY is concentrated in the yolk sac of the egg (72) where it is transported to the embryo during late development by a mechanism similar to that found in mammals (88); thus, it is considered to be of some relevance in maternal passive immunity (53). Passive antibodies, transferred to chicks by hens immunized by gametocyte surface antigens of *Eimeria maxima*, reduced oocyst load in those birds after challenge with sporulated *E. maxima* oocysts (85). Moreover, production of specific antibodies in infected chickens, particularly IgA and IgM, was significantly greater in parasitized areas of the intestine compared with areas devoid of parasites (25). However, the ability of antibodies to limit infection is minimal, if any, because agammaglobulinemic chickens produced by hormonal and chemical bursectomy are resistant to reinfection with coccidia (42,71,96).

Extensive experimental evidence supports the notion that CMI, predominantly mediated by antigen-specific and nonspecific activation of T lymphocytes and macrophages, represents the prevailing component of protective immunity in avian coccidiosis (46,53). For example, changes in intestinal T-cell subpopulations in the duodenum after primary and secondary *Eineria acervulina* infections have been investigated and correlated with disease (44,46,80). These lymphocytes, macrophages, and other effector cells act in harmony to secrete cytokines and proinflammatory molecules, directing the appropriate immune responses to the invading parasite. In contrast to the plethora of mammalian cytokines, only a few chicken homologues have been described, the main ones being interferon (IFN)-γ, transforming growth factor (TGF), tumor necrosis factor, interleukin (IL)-1, IL-2, IL-6, IL-8, and IL-15 as described recently (53). Of late, a number of cytokines, including those of the Th2 type, have been described. These include IL-17 (62); IL-18 (26); IL-16 (63); IL-12 (20); IL-10 (73); and the Th2 type IL-3, IL-4, IL-13, granulocyte macrophage colony stimulating factor (3), and IL-5 (36). Using nucleotide sequence homology and an expressed sequence tags (EST) cDNA library prepared from intestinal intraepithelial lymphocytes (IELs) of *Eimeria*-infected chickens, Min and Lillehoj (62,63) cloned two cDNAs encoding IL-16 and IL-17. Therefore, these cytokines could be participants in the immune responses to coccidiosis. Although not fully characterized, IL-1 association with *Eimeria tenella* and *E. maxima* infections has been described (40). As it stands, Th1 responses seem to be dominant during coccidiosis, as best manifested by proven involvement of IFN-γ (47,48,58,59). Rothwell *et al.* (73) reported an IL-10–induced inhibition of IFN-γ during *E. maxima* infection, suggesting that IL-10 may favor a shift to Th2-type immunity in response to coccidiosis. This furthers our understanding that strong Th1-type, IFN-γ–driven immune responses are the dominant players during *Eimeria* spp. infections.

To better our understanding of the intricate immune response to coccidia, identifying potential genes involved in intestinal health of the chicken becomes essential. This can be achieved by a number of functional genomics tools that include mapping quantitative trait loci (QTL) and microarrays. With DNA marker technology, Zhu *et al.* (97) were able to map QTL associated with resistance to coccidiosis. Conceivably, such loci could hold key genes controlling immunity and resistance to coccidiosis. Global gene expression analysis in *Eimeria*-infected chickens provides major insight into the host protective immune responses to the parasite. With EST sequences from activated T-cell cDNA library, our laboratory identified several genes associated with immune responses to *E. maxima* and *E. acervulina* by DNA array (65). Among those, several interleukins and interferons were upregulated, most notably IL-15 and IFN-γ, after primary infection by either species. As more of these studies are conducted, new information is revealing better comprehension of the innate and adaptive immune responses to pathogens (14,60,77,81,82). Although only a small number of chicken genes have been cloned and completely sequenced, our laboratory has about 10,000 chicken ESTs derived from intestinal IELs of *Eimeria*-infected chickens that are currently available for designing DNA microarrays. Furthermore, the ongoing process of the chicken and *Eimeria* spp. genome projects (76) will undoubtedly uncover fresh and exciting information to progress our grasp of host-parasite

interactions, culminating in novel control approaches that would reduce or eliminate the prophylactic use of anticoccidials.

IMMUNOMODULATION

The gut mucosal system plays a central role in the exclusion and elimination of harmful dietary antigens and enteric pathogens. Nutrition, normal microflora, pathogens, and other factors affect the maintenance of the digestive tract and its associated immune system. However, regulation of immune responses is extremely complex, and complete knowledge of how the immune system responds to infectious agents like *Eimeria* is lacking. Yet one can devise new ways of intervening in the regulation and enhancement of the immune system, particularly by modulating the host's immune response. The term *immunomodulation* is generally used to describe the pharmacologic manipulation of the immune system. This may involve an increase in the magnitude of the immune response, immunostimulation, or a decrease in the magnitude (i.e., immunosuppression). Specific immunomodulation implies a change in the response of the immune system to a particular antigenic stimulus, as achieved by vaccination, whereas nonspecific immunomodulation implies a more fundamental change whereby the state of alertness of the immune system is responsive to a wide range of antigenic stimuli. The principal components of the immune system targeted for immunomodulation include T and B lymphocytes, monocytes and macrophages, granulocytes, and natural killer cells. Cytokines and other antimicrobial secretions are also amenable to immunomodulatory strategies. The final effect will depend on the relative susceptibility of those cell types to the agent used and the contribution they make to nonspecific or specific immune responses. Immunomodulation, therefore, can be used to designate either suppression or augmentation of an immune response. The latter has received much attention in livestock and provides a means of boosting the host's resistance to disease. The necessity of suppressing the function of the immune system has been the result of research studies concerning immunosuppressive factors like toxins and nutrient deficiencies. Various chemicals and biologic substances have been used and evaluated as immunomodulators in poultry; of particular interest are those with known influence on the mucosal physical integrity and immune system, the *Eimeria* spp. infection site. These include, but are not limited to, vitamins and microminerals (e.g., vitamin A); natural products (e.g., betaine); direct-fed microbials (e.g., probiotics); and, more recently, synthetic oligonucleotides with specific rather than nonspecific immunomodulatory effects.

Nutritional immunomodulation. Nutrition plays a significant role in the development and function of the chicken immune system. Essential nutrients such as vitamins may affect both humoral and cell-mediated immune responses. Vitamin A, known for its role in the differentiation of epithelial cells, is essential for maintaining the integrity of mucosal surfaces (11). It is also known to have immunomodulatory effects, and its role in the maintenance of the immune system in a number of animals suggests that its deficiency increases host susceptibility to enteric diseases like coccidiosis (10,16). Indeed, vitamin A deficiency impaired the local immune defenses within the gut lymphoid tissues of broiler chickens (16). This effect was best characterized by a reduction in IEL subpopulations, mainly $CD4^+$ T cells. Alteration in the IEL subpopulations caused by lack of vitamin A lowered the ability of broilers to resist *E. acervulina* infection, resulting in greater oocyst shedding. Furthermore, vitamin A deficiency affected the systemic immune system by reducing the ability of splenic T lymphocytes to respond to *in vitro* mitogen stimulation and also resulted in lower IFN-γ secretion (16). Overall, dietary vitamin A levels can affect gut immunity in broiler chickens, and its deficiency can cause immunosuppression at those sites and result in increased susceptibility to coccidiosis.

Other dietary supplements have been reported to influence immunity to coccidiosis. Betaine, a naturally occurring amino acid derivative, has been investigated as potential enhancing agent against coccidiosis. Klasing *et al.* (32) reported an increase in duodenal IELs of *E. acervulina*–infected chickens as well as an improved functionality of phagocytes. Other studies have shown differential effect on the rate of body weight gain in chickens infected with different *Eimeria* spp., where it was effective only during *E. maxima* infection and not during *E. acervulina* or *E. tenella* (24). When added to salinomycin-treated chickens, betaine significantly reduced invasion by *E. acervulina* and *E. tenella* as compared with invasion in chickens on salinomycin or betaine alone (1).

Probiotics enhance gut defensive mechanisms. The gut microflora constitutes an important component of these first lines of defense in both humans and animals. Probiotic supplementation of the intestinal microflora has been shown to enhance gut defensive mechanisms in poultry (39). Lilly and Stillwell (57) coined the term *probiotic* in 1965, and its definition has subsequently evolved through the years. Perhaps the most appropriate definition is ''probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host'' (74). The development and use of probiotics for farm animals, including poultry, is based on the knowledge that the gut flora is involved in resistance to enteric infections where it has been shown to be involved in protection against a variety of pathogens, including *Escherichia coli* (9,39); *Salmonella* spp. (39,79); *Campylobacter jejuni* (29,79); and, more recently, *Eimeria* spp. (17,18). Therefore, feeding probiotics to animals to maintain a good balance of intestinal microflora could prove effective in the prevention and therapy of such enteric infections by possible modulation of the mucosal immune system and enhancing the host's resistance to enteric pathogens.

Numerous studies have shown disease prevention or immune enhancement resulting from oral feeding of probiotics, but only few reports have examined the specific effects on gut defenses to coccidiosis. We have conducted many studies and demonstrated that a *Lactobacillus*-based probiotic stimulated the local immune system of broiler chickens and improved resistance to *E. acervulina* (17,18,49). The studies involved supplementing broiler chicken diet with a commercial probiotic (Primalac®; Star-Labs/Forage Research, Clarksdale, MO) followed by *E. acervulina* infection. Both local (intestinal) and systemic (serum) immune responses were then assessed by measuring cytokines (namely, IFN-γ and IL-2), antibodies, weight gain, and oocyst shedding (17). Upon examining the effects of feeding the probiotic on the IEL subpopulations and protection against coccidiosis, a significant increase in IEL T-lymphocyte subpopulations expressing the surface markers CD3, CD4, CD8, and α/β- T-cell receptor was observed in probiotic-fed birds compared with control. In general, probiotic-fed chickens produced fourfold fewer oocysts per bird than did untreated controls. Upon testing cytokine and antibody levels in sera and intestinal secretions, the probiotic-fed chickens showed a significantly higher IFN-γ and IL-2 at 3 days postinfection, which was much earlier than shown by the control birds. Probiotic-fed chickens showed lower levels of intestinal antibody against recombinant coccidial antigen than did probiotic-fed chickens. Because probiotic feeding enhanced immune responses to coccidial infection, we investigated its effects on vitamin A–deficient birds. Probiotic-fed chickens shed fewer oocysts than did chickens without probiotic, even in vitamin A–deficient birds, thus confirming improved resistance to coccidiosis in chickens

fed a probiotic supplement. The exact mechanisms underlying the oocyst response are not clear. Early modulation of immune elements in the intestinal epithelium by probiotic bacteria may be one explanation, but more basic research is needed to clarify those effects. A greater understanding of the mechanisms of probiotic-mediated enhancement of intestinal immunity would improve the effectiveness of its use in the field.

CpG oligodeoxynucleotides (ODNs). Short ODNs containing unmethylated CpG motifs have been shown to be effective immunoprotective agents in mammalian models by inducing both innate and adaptive immune responses (37). Recently, CpG ODNs were reported to have both *in vitro* and *in vivo* immunostimulatory effects in domestic animals, including chickens (15,27,30,67,83). In mammalian systems, bacterial DNA displays impressive immunomodulatory action that influences DNA vaccination (28,34). Since its initial discovery (38), ODNs have shown to play a role in host defense, both by stimulating T cells and by inducing cytokines or enhancing innate immunity (33). We have recently identified CpG sequences that activate chicken innate immunity and enhance protective immune response against *Salmonella* spp. and coccidia (15,93). In view of this finding, we are evaluating additional CpG ODNs in our laboratory. One of the ODNs, CpG 2006, had strong stimulatory effects on chicken macrophages as demonstrated by increased proinflammatory cytokine IL-6 secretion, enhanced nitric oxide release, upregulated cell surface marker expression, and increased intracellular bacterial killing (93).

We conducted *in vivo* trials to investigate the immunomodulatory effects of CpG ODNs on disease susceptibility in *E. acervulina*–infected chickens, SC and TK, two genetic chicken lines with different immune responses to *Eimeria* infection: TK is more susceptible than SC. The results showed a CpG effect on body weight gain in both SC and TK chickens but an oocyst shedding effect in TK chickens (15). Only CpG ODN with a phosphorothioate backbone (S-CpG ODN) reduced the number of oocysts shed by TK chickens but not in SC chickens. In previous work, reduced oocyst shedding in TK birds was observed with intravenous CpG ODN injection. However, no clear correlation was between weight gains and oocyst shedding. Enzyme-linked immunosorbent assay results showed higher antibody response in SC chickens injected with the S-CpG ODN. In contrast, no such effect was found in TK birds despite the reduced shedding of oocysts.

Other studies have investigated the *in ovo* immunomodulatory effects of CpG ODNs on disease susceptibility in *E. acervulina*–infected chickens (Dalloul *et al.*, unpubl. data). On day 18 of incubation, specific-pathogen-free chicken embryos were injected with either one of four CpG ODNs, and the hatched chicks were inoculated with 10^4 *E. acervulina* oocysts at 1 wk of age. Two CpG ODNs significantly reduced oocyst shedding, demonstrating that CpG ODNs were effective immunoprotective agents in chickens and could be potentially used for vaccine development to coccidiosis. However, further research into their mode of action and optimization of CpG ODN-induced enhancement of innate immunity in poultry is needed. When coadministered with a recombinant microneme protein (MIC2), both ODNs reduced oocyst shedding; however, only one CpG ODN plus MIC2 consistently improved weight gain. Furthermore, vaccinating with ODN 2006 or MIC2 protein curtailed oocyst shedding but did not enhance weight gain in *E. tenella*–infected birds. Coadministration of CpG ODN and MIC2 did not have an additive effect in reducing the oocyst output; however, it resulted in the highest and lowest Ab response before and after *E. tenella* infection, respectively (Dalloul *et al.*, unpubl. data). Taken together, those trials showed that CpG ODNs administered *in ovo* demonstrated immunoenhancing adjuvant effects after *Eimeria* infections. Current investigations are focused on optimization of vaccination parameters such as adjuvant dosage and delivery schedule.

CONTROL MEASURES AGAINST COCCIDIA

Anticoccidial drugs. Since anticoccidials were introduced in the 1940s, the poultry industry has been largely dependent on their use to prevent and control coccidiosis, and the production of affordable, quality poultry meat owes much to their development (7). They are generally divided into two classes: synthetic drugs and ionophore compounds. Synthetic drugs were introduced first, then the ionophores followed and are now an important component of coccidiosis control. Many existing anticoccidials share a similar chemical composition but carry different trade names depending on the marketing pharmaceutical company (e.g., Salinomycin in Sacox® [Intervet Inc., Millsboro, DE] and Bio-Cox® [Alpharma Animal Health, Fort Lee, NJ]). Some anticoccidials consist of more than one compound, such as Maxiban® (Elanco Animal Health, Greenfield, IN), which is a combination of nicarbazin and narasin. To avoid parasite drug resistance, producers use shuttle programs whereby they rotate the use of different drugs or classes of drugs among different flocks. Despite the availability of several drugs and the application of shuttle programs, resistance has developed to all the anticoccidial drugs introduced so far (6). There is also a lack of new drugs because of the high development costs and stringent testing and regulatory requirements for approval, especially with the short life expectancy of any new drug. Furthermore, public fears of drug residues in the food supply and resistance to antibiotics used in humans led to a recent ban of a number of anticoccidials in Europe (23). Combined, these factors constitute major disincentives to the development of new and more effective drugs, prompting scientists and the industry alike to seek alternative control methods for coccidiosis.

Vaccines against *Eimeria*. *Live vaccines.* Beach and Corl (4) first noted that chickens infected with live coccidia became resistant to challenge with the same parasite, and the first live vaccine (Coccivac®; Schering-Plough, Union, NJ) was made available in the United States in the early 1950s (8). For several decades, live vaccines have been used mostly in breeder stocks and, to a lesser extent, in commercial broilers and replacement hens. This strategy is based on the well-documented protective immunity that develops in chickens after a primary coccidial infection (90). Globally, at least 10 different live vaccine formulations are commercially available. Considerable research and experience have accumulated, and a number of extensive reviews have been published (8,90). Live oocyst vaccines differ in many ways, such as the type of *Eimeria* species (virulent *vs.* attenuated), the drug resistance, the species composition of the product, and the delivery method.

One of the major differences among available live oocyst vaccines is whether the strains of *Eimeria* are virulent or attenuated (90). The virulent or nonattenuated vaccines contain field or laboratory strains that have not been modified in any way, like the Coccivac® and Immucox® vaccines (Vetech Laboratories, Guelph, Ontario), Nobilis® COX ATM, VAC M® (Intervet, Boxmeer, the Netherlands), Inovocox® (Embrex, Inc., Research Triangle, NC), and ADVENT® (Novus International, St. Louis, MO). Some of these vaccines may not contain sufficient numbers of the more pathogenic species to induce long-lasting protective immunity; consequently, their efficacies depend on autoreinfection from recycled parasites. Furthermore, because pathogenicity occasionally predominates over immunogenicity, live vaccines may introduce new species or unexpected pathogens into a flock. On the other hand, attenuated vaccines consist of parasites of artificially reduced virulence,

accomplished mainly either by passaging through embryonated eggs such as *E. tenella* in Livacox® (Biopharm, Jilove u Prahy, Czech Republic) vaccines or by selection for precocity such as the other species of Livacox® vaccines and the Paracox® (Schering-Plough) vaccines. Vaccination with attenuated coccidia parasites avoids some of the problems associated with pathogenic field strains. Among the advantages to using vaccines with precocious strains is that protective immunity is induced without the occasional decline in performance stemming from other more conventional live oocyst formulations. One disadvantage, however, is the higher production cost associated with the lower yield of oocysts in chickens used for generating the vaccine. Aside from this limitation, a number of reports have shown that broilers inoculated with precocious oocyst vaccines performed as well as chickens raised on anticoccidial drugs (13).

Most of these live vaccines contain drug-sensitive strains except for Nobilis® COX ATM and VAC M®, which both contain ionophore-resistant strains of *Eimeria* (90). The inclusion of drug-resistant strains in a vaccine is advantageous because it permits medication with ionophores while allowing immunity to develop. With the exception of one vaccine (VAC M®), all live formulations contain two or more *Eimeria* species. For example, Paracox® consists of all seven species, whereas others have only three to four species (e.g., Coccivac® B, Immucox® C1, ADVENT®). Although the former would protect against any species that arises, it may be more economically feasible to include only those species that are most prevalent and thus likely to cause an outbreak in a given geographic area. However, given that protective immunity against coccidiosis is species specific, administration of a live oocyst vaccine that contains only three to four species may not protect against outbreaks caused by the other species. In addition, some companies include more than one strain of *E. maxima* in a single vaccine (e.g., Paracox® and Nobilis® COX ATM) because of the existing immunovariability among different strains of this species.

In the past few years, a number of different methods have been developed for live oocyst vaccine delivery (8,90). Among the first were suspension in drinking water and spraying directly on the feed, which have largely been replaced with hatchery spray administration to day-old chicks. The first method was intraocular delivery, which sprayed the oocyst suspension directly into the eye. The oocysts would pass down the nasolacrimal duct and reach the intestine via the oropharynx. This method, however, required skilled labor and has fallen out of use in the United States. Spray cabinet administration is another hatchery application by which the vaccine is sprayed over chick trays. The oocysts are suspended in a colored dye that has the dual advantage of allowing hatcheries to visually evaluate the success of the procedure and also stimulate chicks to take up the oocysts by preening themselves and each other. Another successful delivery method is the incorporation of vaccine oocysts in a colored gel (Immucox®) that is placed in chick trays at the hatchery or on feed trays in the poultry house. The chicks ingest the gel and thereby take up the oocysts, which results in a patent infection and development of immunity. Danforth (19) compared four different methods of delivery of the Immucox® vaccine (gel delivery, crop gavage, spray cabinet, and slurry delivery) and found gel delivery to be superior to the others, even though all four resulted in significant protection. Recently, a proprietary device was developed to deliver a live vaccine by intra-yolk sac administration (21); however, this method has yet to be adopted by any available vaccine. The most recent advance in live oocyst delivery is the *in ovo* injection of sporulated oocysts into 18-day-old embryonated eggs (Inovocox®). Several studies have shown that *in ovo* immunization of broilers with *Eimeria* spp. sporozoites, sporocysts, or oocysts provide protection against challenge infection (86,87). *In ovo* administration of live oocyst vaccines has several distinct advantages, including the increased accuracy and repeatability of vaccine delivery. Although this particular vaccine is in the final stages before marketing, other products are being developed for *in ovo* vaccination.

Recombinant vaccines. Current control methods consist of chemical prophylaxis or live parasite vaccination. For reasons of safety, cost, and emergence of drug-resistant *Eimeria* strains, much research has focused on recombinant vaccination strategies as potential alternative methods of disease control. The conception of genetic vaccines emerged from the observation that injection of naked plasmid DNA resulted in transfection of murine muscle cells and production of the plasmid-encoded protein β-galactosidase (91). Later, analyzing the mechanism of operation made it clear that DNA not only is simply a vehicle to ensure protein production in transfected cells, but it also has intrinsic adjuvant properties because of the presence of immunostimulatory CpG dinucleotide in the backbone of bacterial DNA (37). A number of recent studies have presented promising evidence of effective recombinant protein and DNA vaccination against coccidiosis.

The identification of antigens specific to parasite life cycle stages conveying protective immunity is a pivotal step in subunit vaccine development. In *Eimeria* spp., recombinant forms of both parasite surface antigens and internal antigens have been examined as vaccine candidates (61,75). Belli *et al.* (5) cloned and expressed two recombinant proteins of the genes gam56 and gam82, encoding the immunodominant components of a commercial subunit vaccine called CoxAbic® (ABIC Veterinary Products, Beit Shemesh, Israel) (not available in the United States) derived from *E. maxima* gametocytes. This vaccine has been shown to provide partial protection against *E. acervulina*, *E. maxima*, and *E. tenella* (84), but its production is both laborious and costly (5). After multiple immunizations with the recombinant proteins, alone or in combination, breeding hens elicited a dose-dependent antibody response indicative of similar antigenic and immunogenic properties to the native protein vaccine. These proteins can be potentially used in developing recombinant vaccine at lower costs than with CoxAbic®. Our laboratory recently tested a purified *E. acervulina* recombinant protein (3-1E) to vaccinate chickens *in ovo* against coccidiosis both alone and with expression plasmids encoding the IL-1, IL-2, IL-6, IL-8, IL-15, IL-16, IL-17, IL-18, or IFN-γ genes (22). We showed that *in ovo* vaccination with 3-1E protein enhanced protective immunity against *E. acervulina* infection as measured by reduced fecal oocyst shedding and increased body weight gain compared with non-vaccinated controls. Also, covaccination with 3-1E plus the IL-2, IL-15, IL-17, IL-18, or IFN-γ genes further reduced the oocyst output beyond that induced by 3-1E alone. A second potential recombinant protein was evaluated as a coccidiosis vaccine; its gene (EtMIC2) was cloned, the encoded protein expressed and purified, and the efficacy of *in ovo* immunization to protect against *Eimeria* infections was determined (50). We demonstrated that *in ovo* vaccination with the recombinant EtMIC2 protein induced significantly higher antibody responses, lower oocyst fecal shedding, and increased weight gains after *E. tenella* infection compared with negative controls. Furthermore, combined embryo immunization with the EtMIC2 protein plus chicken cytokine or chemokine genes (IL-8, IL-16, TGF-β4, and lymphotactin) demonstrated enhanced protection compared with vaccination with EtMIC2 alone (50). Taken together, these results provide the first evidence that *in ovo* vaccination with the recombinant 3-1E and EtMIC2 *Eimeria* spp. proteins induced protective intestinal immunity against coccidiosis. Furthermore, their protective effects were enhanced by coadministration of genes encoding immune-

related cytokines, paving the way for a potentially effective method to control coccidiosis.

On the other hand, DNA vaccines use genes encoding immunogenic proteins of pathogens rather than the proteins themselves. They are administered directly in conjunction with appropriate regulatory elements (e.g., promoters, enhancers) permitting the encoded protein to be expressed in its native form and thereby to be recognized by the host's immune system in a manner that simulates natural infection. Kopko *et al.* (35) were able to ligate SO7′, a refractile body encoding gene derived from *E. tenella* sporozoites, to the mammalian expression vector pcDNA3. After intramuscular injection of the pcDNA3-SO7′ construct and subsequent *E. tenella* challenge, significant protection against cecal lesions and weight loss was achieved. Recently, Wu *et al.* (92) constructed two DNA vaccines based on antigens present on *E. tenella* sporozoites. After DNA immunization and *E. tenella* challenge, the authors reported reduced oocyst shedding as well as decreased weight loss. Lillehoj *et al.* (48) observed immune protection manifested by significantly reduced fecal oocyst shedding in chickens vaccinated subcutaneously with a cDNA encoding *E. acervulina* 3-1E protein. Further protection was obtained when the 3-1E cDNA was administered in conjunction with cDNAs encoding chicken IFN-γ or IL-2. Later, Min *et al.* (64) examined the effects of injecting a plasmid encoding the 3-1E gene in combination with a plasmid encoding IL-1β, IL-2, IL-8, IL-15, IFN-α, IFN-γ, TGF-β4, or lymphotactin and delivered twice subcutaneously to chickens, followed by challenge 1 wk later. Body weight loss was significantly reduced in chickens given the DNA vaccine with the IFN-α or the lymphotactin-encoding plasmid, whereas parasite replication was reduced in chickens injected with the IL-8, lymphotactin, IFN-γ, IL-15, TGF-β4, or IL-1β-encoding plasmids, compared with chickens vaccinated with the 3-1E DNA vaccine alone. Furthermore, the groups of chickens that were given the IL-8 or IL-15 genes had significantly increased numbers of CD3+ T cells compared with the other groups. More recently, Lillehoj *et al.* (51) used a similar scheme to inject the 3-1E and cytokine encoding plasmids *in ovo* and assess its protection against *E. acervulina* infection. *In ovo* vaccination with the 3-1E gene generated an antibody response against the expressed parasite protein that was enhanced by covaccination with the IL-1, IL-2, IL-15, or IFN-γ genes. *In ovo* vaccination with 3-1E demonstrated protective immunity against *E. acervulina* infection as measured by reduced oocyst shedding and improved body weight gain compared with nonvaccinated controls. The data also showed that covaccination of 3-1E with the IL-2, IL-15, or IFN-γ genes further curtailed oocyst output and exceeded weight gain beyond that induced by 3-1E alone.

CONCLUSION

The need to continue to seek more effective ways to minimize the impact of poultry coccidiosis is a must in an ever-growing worldwide industry. A number of potential strategies are presented, including the use of immunomodulators (e.g., nutritional and probiotics), adjuvants, and recent advances in recombinant vaccine development. Enhancing immunity with the intent of augmenting resistance to parasitism by *Eimeria* spp. is a goal and should at least alleviate the economic burden carried by coccidiosis. This could be achieved by immunomodulation, which may provide a potent mechanism by which we enhance the ability of birds to better withstand disease. Furthermore, the lack of effective coccidiosis vaccines, along with the emergence of drug-resistant strains of *Eimeria*, has prompted poultry scientists to investigate alternative vaccination strategies in terms of both new and novel vaccines and delivery methods. One such avenue is recombinant vaccination, which, when coupled with *in ovo* delivery along with appropriate adjuvants, offers a promising means of controlling coccidiosis. However, performance of such vaccines will have to withstand the test of evaluation in the commercial setting.

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